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Detection of new biased agonists for the serotonin 5-HT_{2A} receptor: modeling and experimental validation

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Running title: New biased agonists for serotonin 5-HT_{2A} receptors

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Text pages: 29

Tables: 1

Figures: 3

References: 34

Abstract: 134

Introduction: 621

Discussion: 377

Abbreviations: AA, arachidonic acid; GPCR, G protein-coupled receptor; IP, inositol phosphate; MetI, 3-(aminoethyl)1-methylindol-5-ol; MetT, 5-methyltryptamine; NitroI, 5-Nitro-1H-indole-3-ethanamine; 2C-N, 2,5-dimethoxy-4-nitrophenethylamine.

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Abstract

Detection of biased agonists for the serotonin 5-HT2A receptor can guide the discovery of safer and more efficient antipsychotic drugs. However, the rational design of such drugs has been hampered by the difficulty to detect the impact of small structural changes on signaling bias. To overcome these difficulties, we characterized the dynamics of ligand-receptor interactions of known biased and balanced agonists using molecular dynamics simulations. Our analysis revealed that interactions with residues S5.46 and N6.55 discriminate compounds with different functional selectivity. Based on our computational predictions, we selected three derivatives of the natural balanced ligand serotonin and experimentally validated their ability to act as biased agonists. Remarkably, our approach yielded compounds promoting an unprecedented level of signaling bias at the 5-HT2AR, which could help interrogating the importance of particular pathways in conditions like schizophrenia.

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Introduction

Serotonin 5-HT_{2A} receptors are G protein-coupled receptors (GPCRs) targeted by hallucinogenic drugs of abuse (Nichols, 2004) as well as by second generation antipsychotic drugs (Meltzer, 1999), which function as antagonists at these receptors (González-Maeso and Sealfon, 2009). However, the basis of serotonin 5-HT_{2A} receptor functioning is still not fully understood. Past studies on this receptor revealed that it can be differentially modulated by diverse agonists. Specifically, 5-HT_{2A} receptors were one of the first GPCRs for which functional selectivity was described (Berg *et al.*, 1998). Functional selectivity allows some GPCRs to preferentially signal through a signaling pathway over another when they interact with certain ligands, namely biased agonists (Urban *et al.*, 2007). Biased agonists are suggested to promote the stabilization of distinct receptor activation states with a preference to couple to a given signal transducer and thus favor signaling through a particular pathway (Park, 2012). Ever since the first description of this class of compounds, they were considered likely drug candidates (Whalen *et al.*, 2011). Biased agonists hold a big potential as new generation drugs with increased efficacy and safety (Martí-Solano *et al.*, 2013) by modulating pathways implicated in disease, while sparing other non-related cellular processes regulated by the activation of the same receptor.

More than a decade ago, Berg *et al.* described the different ability of some ligands to trigger two independent signaling responses at the 5-HT_{2A} receptor: the accumulation of inositol phosphate (IP) and the release of arachidonic acid (AA) (Berg *et al.*, 1998). Implication of these two pathways in processes such as the generation of hallucinogenic effects is still not completely

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understood (González-Maeso *et al.*, 2007). In parallel, recent studies on the inactivation of the 5-HT_{2A} receptor by antipsychotic drugs have pointed to an unwanted silencing effect on the transcription of the mGlu2 receptor, suggesting that full receptor inactivation could be counterproductive for the treatment of schizophrenia (Kurita *et al.*, 2012). For these reasons, obtaining biased agonists capable of selectively activating each of these signaling pathways could help explore to which extent they are implicated in the aforementioned pathophysiological processes. This knowledge could, in turn, suggest new strategies for the design of more efficient drugs targeting the 5-HT_{2A} receptor.

At present, however, the rational design of biased agonists is hampered by the fact that the structural basis of functional selectivity is not fully understood. The problem arises partially from the challenge of attributing structural differences of agonist binding to distinct signaling states of the same receptor, which can be relatively subtle. Given the ability of GPCRs to explore different activation states, a single static picture of an activated receptor may not be enough to characterize contacts with agonists that promote different types of signaling bias. Therefore, a dynamic view of ligand-receptor interactions could add important information to understand the phenomenon of biased agonism. Recent advances in molecular dynamics (MD) simulations, which are currently used to study processes such as GPCR activation-inactivation (Dror *et al.*, 2011) or stabilization of different receptor populations by agonists and inverse agonists (Nygaard *et al.*, 2013), provide a powerful tool for analyzing the structural basis of biased agonism at an adequate structural and temporal resolution.

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Our study aims to learn from the dynamics of ligand-receptor interaction of known biased agonists, and apply this knowledge to the design of ligands with a tailored biased signaling profile. In order to assess this approach we have applied extensive MD simulations with an accumulated time of 10 μs to study the structural determinants of biased agonism at the 5-HT_{2A} receptor from a dynamic perspective (see Supplemental Table 1 for details). Our study revealed ligand features as well as relevant hotspots within ligand interaction profiles that are related to signaling bias. By exploiting this structural knowledge, we have predicted compounds with the potential to behave as biased agonists. Importantly, experimental characterization of these compounds verified their biased nature and confirms the value of MD simulations for rationally detecting ligands promoting tailored signaling outcomes, which can provide a starting point for the design of new antipsychotic therapies.

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Materials and Methods

Homology modeling and ligand docking

Even if X-ray crystal structures of serotonin receptors have become recently available (PDB IDs 4IAR and 4IB4), the fact that these receptors have been crystallized in intermediate activation states (Wacker *et al.*, 2013), which cannot accommodate a G protein (see Supplemental Figure 6), led us to select the structure of the β2-adrenergic receptor in complex with G_s as the starting template to ensure simulation of a fully activated receptor. The modeling protocol included alignment of the sequence of the serotonin 5-HT_{2A} receptor to the one of the β2-adrenergic receptor in complex with G_s (PBD ID 3SN6) using MOE software (Molecular Operating Environment (MOE) software, http://www.chemcomp.com/software.htm). A structural model of

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the receptor was then built using MODELLER software (Latek *et al.*, 2013). The resulting structures were optimized using the AMBER12:EHT force field (Case, 2012) in the MOE software. The stereochemical quality of the model was evaluated with PROCHECK (Laskowski *et al.*, 1993). The mutant receptor was obtained by using the Mutate plugin of MOE. After being analyzed with MoKa (Milletti and Vulpetti, 2010), the ligands were docked using GOLD software (Verdonk *et al.*, 2003) and the conformational space of the ligands was explored with the Low Mode Search function of MOE using the AMBER12:EHT force field (for further methodological information please refer to the Supplemental Methods).

System preparation and molecular dynamics simulations

Complexes resulting from the previous step were subsequently used to build the initial models for MD simulations (PDB and topology files of ligand - 5-HT2A receptor complexes are available for serotonin (Data Supplement 1 and Data Supplement 2), 2C-N (Data Supplement 3 and Data Supplement 4), MetT (Data Supplement 5 and Data Supplement 6), MetI (Data Supplement 7 and Data Supplement 8), and NitroI (Data Supplement 9 and Data Supplement 10). First, the protonation state of titratable groups was predicted for a pH value at 7.4 based on PROPKA (Li *et al.*, 2005) using the implemented prediction tool of the MOE package. Subsequently, in order to place both receptors into a membrane bilayer, a hole was generated by removing POPC molecules of a pre-equilibrated palmitoyloleoylphosphatidylcholine bilayer generated using the CHARMM-GUI Membrane Builder (Jo *et al.*, 2009). Lipids which were in close contact with the protein atoms (<1 Å distance from any protein atoms) were deleted. Finally, the coordinates for water and ions were generated using the solvate and autoionize

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modules of VMD 1.9.1 (Humphrey *et al.*, 1996). The ionic strength was kept at 0.15 M by NaCl and we used the TIP3 water model. The all-atom models of each system were generated by using the Amber03 force-field parameters and the different ligands were parameterized using Antechamber from AmberTools 11 (Case, 2012). Simulations were performed using ACEMD using the protocol described in the Supplemental Methods (Harvey and De Fabritiis, 2009). Simulations were performed for individually generated starting structures and each ligand-receptor complex was run eight times for 250 ns. Analysis of ligand-receptor interactions was performed by considering residues at a distance smaller or equal to 3 Å of each ligand across the simulation time.

Drugs and reagents

[^{3}H]*myo*-Inositol (20.3 Ci/mmol) and [^{14}C] arachidonic acid (57.1 mCi/mmol) were purchased from PerkinElmer Life Science (Waltham, Massachusetts). 3-(2-aminoethyl)-1-methyl-1H-indol-5-ol was purchased from Otava Ltd (Vaughan, Ontario). serotonin, 5-methyltryptamine and 2-(5-nitro-1H-indol -3-YL) ethanamine were purchased from Sigma Aldrich (St. Louis, MO). RNA Binding YSi SPA Beads and OptiPhaseSupermix Cocktail were purchased from Perkin Elmer. Albumin, Fraction V fatty acid free was purchased from Roche (Basel, Switzerland). All other reagents were purchased from Sigma Aldrich.

Cell culture

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Chinese hamster ovary cells stably expressing human 5-HT_{2A} receptor at a density of ≈ 200 fmol/mg protein (CHO-FA4 cells, previously used in 2C-N biased agonism determination by Moya *et al.* (Moya *et al.*, 2007)) were maintained in standard tissue culture plates (150 mm in diameter) in Dulbecco's modified Eagle's medium-F12 (GIBCO) supplemented with 10% (v/v) fetal bovine serum (FBS; Sigma Aldrich), 1% L-glutamine (Sigma Aldrich), 100U/ml Penicillin/0.1mg/ml Streptomycin (Sigma Aldrich) and 300 µg/ml hygromycin (Invitrogen). Cells were grown at 37ºC in a 5% CO_2 humidified atmosphere.

Competition Binding in Human 5-HT_{2A} receptors

Serotonin 5-HT_{2A} receptor competition binding experiments were carried out in membranes from CHO-5HT_{2A} cells. On the day of the assay, membranes were defrosted and re-suspended in binding buffer (50 mM Tris-HCl, pH 7.5). Each reaction well of a 96-well plate, prepared in duplicate, contained 80 µg of protein, 1 nM [^{3}H]ketanserin (50.3 Ci/mmol, PerkinElmer), and compounds in various of concentrations. Non-specific binding was determined in the presence of 1µM methysergide (Sigma Aldrich). The reaction mixture was incubated at 37°C for 30 min, after which samples were transferred to a multiscreen FB 96-well plate (Millipore, Madrid, Spain), filtered, and washed six times with 250 µl wash buffer (50 mM Tris-HCl, pH 6.6), before measuring in a microplate beta scintillation counter (Microbeta Trilux, PerkinElmer, Madrid, Spain).

Measurement of IP accumulation and AA release in CHO-FA4 cells expressing 5-HT_{2A} receptors

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Cells were seeded into 96-well tissue culture plates at a density of $2x10^4$ cells/cm^2. After 24 h, medium was replaced by serum-free medium with 10 µCi/ml *myo*-[^{3}H]-inositol (20.3 Ci/mmol) for 24 h and 0.2 µCi/ml [^{14}C] arachidonic acid (57.1 mCi/mmol) for 4 h at 37ºC. Measurement of IP accumulation and AA release were made simultaneously from the same well (Berg *et al.*, 1998, 1999). After the labeling period, cells were washed for 10 min at 37ºC with Hanks' balanced salt solution supplemented with 20 mM HEPES, 20 mMLiCl and 2% fatty acid free BSA (experimental medium). After washing, cells were incubated for 20 min with experimental medium at 37ºC containing vehicle or the indicated concentrations of drugs. At the end of the incubation time, aliquots of 90 µl of media were added to flexiplate with 150 µl OpthiPhase for the measurement of [^{14}C] which corresponds to AA release. The remaining medium was discarded and 200 µl of 100 mM formic acid was added to the cells for 30 min at 4ºC, aliquots of 20 µl were added to flexiplate with 80 µl of a solution RNA Binding YSi SPA Beads for measuring accumulation of [^{3}H] IPs from the cells (IP_1, IP_2, and IP_3, collectively referred to as IP). Radioactivity was quantified with a liquid scintillation counter WALLAC MicrobetaTriLux 1450-023. The same procedure was used in a CHO WT cell line to assess dependency on the 5-HT_{2A} receptor for AA and IP signaling activation.

Pharmacological data analysis

Stimulation response parameters were calculated with Prism 4.0 software applying a four parameters logistic equation. In the case of MetI, the fact that this ligand was not completely biased for one pathway led us to calculate a bias factor using an equiactive comparison. This method gives a good estimate of bias when the dissociation constant for a ligand is not known

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(Rajagopal *et al.*, 2011). This comparison has proven to be useful provided that the ligand is not a partial agonist or a strongly biased compound, as in the present case. Therefore, using this approach, comparison between the ligand and a reference (in this case, serotonin) provides a bias factor (β) for pathway P1 versus P2, which can be calculated as follows:

$$\beta = \log\left(\left(\frac{E_{\max,P1}}{EC_{50,P1}}\frac{EC_{50,P2}}{E_{\max,P2}}\right)_{lig} \times \left(\frac{E_{\max,P2}}{EC_{50,P2}}\frac{EC_{50,P1}}{E_{\max,P1}}\right)_{ref}\right)$$

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Results

Assessing the interaction preferences of known compounds

In order to characterize the structural determinants of biased signaling at the 5-HT_{2A} receptor, we started analyzing two representative compounds: serotonin, the natural ligand, which produces a balanced response for the two studied pathways, and 2,5-dimethoxy-4-nitrophenethylamine (2C-N), a compound capable of partially stimulating AA release but lacking efficacy for IP accumulation (Moya *et al.*, 2007). Both compounds, the natural ligand serotonin (used as a control for balanced agonism) and 2C-N, were docked into a fully activated model of the serotonin 5-HT2A receptor. Notably, the modeling approach used to obtain these ligand-receptor complexes - detailed in the Materials and Methods section - has previously proven to be highly effective in predicting high-resolution ligand-receptor complexes for related targets (Obiol-Pardo *et al.*, 2011). The resulting complexes were embedded into a hydrated lipid bilayer, ionized to a physiological concentration and subjected to extensive molecular dynamics simulations. Given the importance of appropriately sampling ligand-receptor conformational space and of retaining an activated receptor state for the study of biased agonism, we prioritized the use of independent replicates over the study of single prolonged simulations, which would have likely resulted in receptor inactivation as observed for other GPCRs (Dror *et al.*, 2011). In addition, simulations in which the receptor progressed to a fully inactivated state (assessed by ionic lock closure) were discarded from the analysis. Ultimately, the resulting simulations used for both ligands consist of 8 independent replicates per ligand-receptor system amounting to a total simulation time of 4 μs (see Supplemental Table 1).

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A structural analysis of the simulation reveals that the studied compounds can sample several positions within the orthosteric binding pocket (Figure 1, middle panel). Both ligands establish previously known interactions with the receptor and adopt a general conformation which is in line with previous serotonin binding models (Ebersole *et al.*, 2003). For instance, they form a well-described salt bridge between their positively charged nitrogen and the carboxylate of residue D3.32 (residue numbers follow the Ballesteros-Weinstein numbering scheme (Ballesteros and Weinstein, 1995)). In addition, they establish common hydrophobic contacts between their aromatic regions and residue V3.33. In general terms, taking into account the complete interaction list of both ligands, it would not be possible to establish a differential interaction pattern. However, if we analyze the preferred interactions of both ligands over the whole simulation time, we can find interesting differences between both compounds. Hence, considering the top 5 interactions for each ligand (Figure 1, bottom panel and Supplemental Figure 1), the balanced natural ligand, serotonin, adopts two main stabilizing interactions in the form of two hydrogen bonds: one hydrogen bond is formed between the nitrogen of its indole ring and residue S5.46, whereas the other one is established between its hydroxyl substituent and residue N6.55. It is worth mentioning that N6.55 can also form a hydrogen bond with residue S5.43. Previous experimental evidence suggests that S5.43 is able to establish indirect interactions with different serotonergic agonists (Braden and Nichols, 2007). This would be in line with our ligand binding mode in which S5.43 does not show direct contacts with serotonin but indirect ones via N6.55. In contrast to serotonin, the biased compound 2C-N enters deeply into the receptor and interacts frequently with residue F6.51. Besides, the methoxy substituent present in this compound reaches higher towards the extracellular receptor opening and interacts

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with residue V5.40 of helix 5. Interestingly, within its top 5 interactions, we find that 2C-N is capable of forming a contact between its nitro group and residue N6.55 in helix 6, which is also observed for serotonin the natural ligand (Figure 1, bottom left panel). This observation suggested that interaction with N6.55 could be responsible for the activation of the AA pathway, as both serotonin and 2C-N interact with this residue and promote AA signaling. In this sense, these result points to position 6.55 as a possible hotspot determining AA over IP signaling. This is in line with site-directed mutagenesis studies at position 6.55 in other aminergic GPCRs. These studies revealed the influence of this position on biased signaling related to differential G protein coupling (Tschammer *et al.*, 2011; Fowler *et al.*, 2012). In parallel, the finding that serotonin establishes an interaction with residue S5.46 in helix 5, which is not seen in the dynamic binding profile of 2C-N, could justify the biased nature of the latter. Mutations in this position in receptors transfected in HEK293 cells, which have shown somewhat conflicting results regarding the binding mode of different tryptamines, highlight the importance of this interaction in the case of serotonin and call for a deeper characterization in our studied system (Braden and Nichols, 2007).

Considering N6.55 vs. S5.46 interaction preferences to propose new biased agonists

Taken together, observations on the binding preferences of known balanced and biased agonists, and especially of the importance of interaction with residues N6.55 and S5.46, led us to suggest that biased agonism at the 5-HT_{2A} receptor is determined as follows: ligand interaction with residue N6.55 in helix 6 favors the stabilization of receptor conformations with a preference to signal through the AA pathway, while interaction with S5.46 in helix 5 is responsible for

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facilitating signaling through the IP pathway. At this point, the most challenging task was to apply this structural knowledge for the experimental detection of new biased agonists. Such detection would represent an important milestone to validate our previous observations based on molecular dynamics simulations. We hypothesized that, based on the above defined requirements, we could introduce structural modifications into the balanced natural agonist serotonin turning it into a biased compound with a tailored signaling behavior. To test this hypothesis, we searched for novel, commercially available and previously uncharacterized ligands for biased agonism that contain a tryptamine scaffold with the potential to interact with residues N6.55 or S5.46. Our search yielded three interesting compounds. The first selected compound is 3-(aminoethyl)1-methylindol-5-ol (MetI, Figure 2, upper panel). Compared with serotonin, this compound has a methyl substitution at the amine of the indol group, which, in principle, would diminish the capacity for hydrogen bonding with residue S5.46 in helix 5, but would still allow interaction with N6.55, therefore promoting AA over IP signaling. The second candidate, 5-methyltryptamine (MetT, Figure 2, upper panel), has a methyl substitution in the position occupied by the hydroxyl group in serotonin. According to our hypothesis, this compound should show a decreased ability to form a hydrogen bond with residue N6.55 hence making it a biased agonist by promoting IP over AA signaling. Finally, in order to assess in a more refined way the ligand determinants related to functional selectivity, we selected a third compound, namely 5-Nitro-1H-indole-3-ethanamine (NitroI, Figure 2, upper panel, right). This last compound preserves the amine of the indol group found in serotonin but has a nitro group substitution in the position occupied by the hydroxyl group of the natural ligand. In this way, this compound allows assessing the importance of the nitro group present in 2C-N, for interaction

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with residue N6.55. If the effect of the nitro group interaction is equivalent to the one established by the hydroxyl group of serotonin, NitroI should be able to signal through both pathways.

Upon selection of these three new biased agonist candidates, we undertook a new set of MD simulations in order to characterize their behavior inside of the serotonin 5-HT_{2A} receptor binding pocket. We conducted the same protocol as the one previously applied for serotonin and 2C-N. The conformational space sampled by all three compounds is shown in the middle panel of Figure 2. As in the case of serotonin and 2C-N, considering an extended list of ligand-receptor contacts (Supplemental Figure 2) does not allow us to discriminate differential interaction patterns among the proposed biased ligands. Notably, a structural analysis of the overall receptor conformational space of the 5-HT_{2A} receptor in complex with our studied ligands (Supplemental Figure 3), despite showing some differences, does not allow discriminating different signaling signatures either. Conversely, analysis of top 5 ligand-receptor interactions reveals some expected differences in ligand interaction preferences. In detail, main interactions such as the salt bridge between the protonated nitrogen and D3.32 as well as hydrophobic contacts with V3.33 were observed among the selected compounds (Figure 2, bottom panel and Supplemental Figure 2). Assessment of the top 5 interactions for compounds MetI and MetT also shows differences in interaction with defined hotspots for biased signaling. In this context, our simulations reveal a preference of MetI to interact with residue N6.55 (Figure 2, bottom panel, left). This behavior is in agreement with our initial prediction that MetI should especially promote signaling through the AA pathway. Conversely, MetT favors interaction with S5.46 in helix 5 (Figure 2, middle panel), and therefore is predicted to stimulate signaling through the IP pathway. Interestingly, an unexpected behavior was observed for the third compound, NitroI. Even if this compound is able

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to interact at times with position N6.55 through its nitro group, analysis of the total simulation time shows that this is not a within the top 5 interactions (Figure 2, bottom panel) and that NitroI clearly favors hydrogen bonding with residue S5.46. According to our defined criteria for biased signaling, this observed interaction pattern indicates that NitroI should promote IP over AA signaling. All in all, our dynamic analysis predicts that MetI favors AA signaling whereas MetT and NitroI signal preferentially via the IP pathway.

Experimental validation of biased agonism for new compounds

To validate the accuracy of our computational predictions, we experimentally determined their levels of signaling for the AA and the IP pathway (Figure 3 and Table 1). In a first step, we confirmed that MetI, MetT and NitroI bind specifically to the 5-HT_{2A} receptor with binding affinity constants (Ki) of 3.25, 0.86 and 2.05 µM respectively (Supplemental Figure 4). Besides that, AA and IP stimulation is not observed in the parental cell line either in the presence of serotonin or the new tested compounds (Supplemental Figure 5), indicating that stimulation of these pathways depends on ligand binding to the 5-HT_{2A} receptor. Regarding functional selectivity, in line with our computational prediction, our first tested compound, MetI, shows a preference to signal through the AA pathway over the IP one (Figure 3 and Table 1). This can be deduced from calculating its bias factor, which quantifies the relative stabilization of one signaling state over another compared with the reference agonist (Rajagopal *et al.*, 2011) (please refer to the Materials and Methods section for a description of ligand bias calculation). Comparison of MetI with the natural ligand serotonin gives a bias factor of 1.77, indicating that MetI activates 17.7 times better the AA over the IP pathway than serotonin. This first validation

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clearly demonstrates the ability of our model to rationally tune the balanced signaling stimulated by serotonin into an AA signaling preference. Even more striking are the results obtained for MetT. On top of showing the predicted bias for IP signaling, this compound does not have any detectable ability to promote signaling through the AA pathway at our tested concentrations, thus behaving as a highly biased agonist for the IP pathway (Table 1). NitroI, also follows its predicted pattern, and, remarkably, it is capable of behaving as a full agonist for IP signaling at a nanomolar scale while not triggering the AA pathway. To our knowledge, this level of bias is unprecedented at this receptor and would make this last compound a particularly interesting tool to explore serotonin 5-HT_{2A} receptor pharmacology.

Discussion

In our work, we have used extensive MD simulations to learn from the dynamics of ligand-receptor interactions of biased agonists. This dynamic insight provides a thorough sampling of ligand binding preferences capable of discriminating different types of receptor agonists. In our experience, this discrimination would have been difficult if only their docking poses had been considered. Our simulations highlight the importance of contacts with particular receptor hotspots for biased agonism, namely N6.55 in the case of AA signaling and S5.46 in the case of IP signaling. Based on this knowledge, we have discovered new biased ligands of unprecedented efficacy by tuning the structure of the balanced natural ligand serotonin. Experimental validation of the proposed ligands has proven the power of characterizing dynamics of ligand-receptor interactions to obtain ligands with a tailored biased signaling profile. This study, however, poses interesting questions on the process of functional selectivity

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at the 5-HT_{2A} receptor that go beyond ligand-receptor interactions. Given that in our study we were only able to obtain compounds with very high levels of bias for IP signaling (MetT and NitroI), the question remains of how feasible it is to obtain this kind of agonists for the AA pathway. This could be a complicated mission, in case receptor conformations related to differential coupling overlap in such a way that when activating the AA pathway there will always be a receptor population capable of triggering IP signaling. This calls for a deeper structural characterization of diverse receptor states coupled to specific signaling transducers. In parallel, the applicability of our model could be further extended by the incorporation of additional 5-HT_{2A} receptor agonists possessing significantly different chemical scaffolds than the ones considered in this work. Further experimental and computational studies will be needed to solve these questions. In particular, experimental structural information on receptors coupled to different G proteins would shed light on the overall receptor architecture required for differential coupling. This information would enrich studies as the current one, as interaction with different biased agonists in the absence of a G protein is not considered enough to stabilize particular receptor signaling states (Rasmussen *et al.*, 2011; Thanawala *et al.*, 2014). Nonetheless, results presented in this work highlight the potential of ligand-receptor dynamics simulations to rationalize biased signaling determinants. In our particular case, the obtained biased agonists could represent valuable tools to interrogate particular signaling pathways, as well as inspire the development of new drug candidates with improved efficacy and safety profiles for the treatment of conditions such as schizophrenia.

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Authorship Contributions

Participated in research design: Martí-Solano, Sanz, Brea, Pastor, and Selent

Conducted experiments: Martí-Solano, Iglesias, and Selent

Contributed new reagents or analytic tools: de Fabritiis, and Selent

Performed data analysis: Martí-Solano, Brea, and Selent

Wrote or contributed to the writing of the manuscript: Martí-Solano, Sanz, Brea, Loza, Pastor, and Selent

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Footnotes

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Legends for Figures

Figure 1. Analysis the dynamic binding profile of known biased and balanced ligands. Upper panels: structures of the starting compounds (serotonin: orange and 2CN: beige). Middle panels: conformational space explored by each ligand as a superposition of 1 every 20 frames per trajectory. Bottom panels: analysis of preferred ligand-receptor interactions. Key residues implicated in ligand-receptor hydrogen bonding are highlighted in red and bold. Hydrogen bonding is indicated as red dashed lines.

Figure 2. Analysis the dynamic binding profile of potential biased ligands. Upper panels: structures of proposed biased agonists (MetI: purple, MetT: yellow and NitroI: magenta). Middle panels: conformational space explored by each ligand as a superposition of 1 every 20 frames per trajectory. Bottom panels: analysis of preferred ligand-receptor interactions. Key residues implicated in ligand-receptor hydrogen bonding are highlighted in red and bold. Hydrogen bonding is indicated as red dashed lines.

Figure 3. Results from pharmacological characterization of novel compounds. Data correspond to the mean of 3 independent experiments with duplicate observations for each experiment.

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Serotonin - 5-HT2A receptor complex – PDB and topology files of the starting complex.

2C-N - 5-HT2A receptor complex – PDB and topology files of the starting complex.

MetT - **- 5-HT2A receptor complex** – PDB and topology files of the starting complex.

MetI - **- 5-HT2A receptor complex** – PDB and topology files of the starting complex.

NitroI - **- 5-HT2A receptor complex** – PDB and topology files of the starting complex.

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Ligand	EC_{50} AA signaling [a]	E_{max} AA signaling [a]	EC_{50} IP signaling [a]	E_{max} IP signaling [a]
Serotonin	0.433±0.160	100±6	0.122±0.030	100±3
MetI	0.210±0.023	100±6	3.520±0.171	100±5
MetT	-		0.437±0.072	86±2
NitroI	-		0.491±0.051	104±2

Table 1. Pharmacological data of novel compounds for the IP and AA pathways at human 5-HT_{2A} receptors. [a] Data correspond to mean±SD EC_{50} values (μM) of 3 independent experiments with duplicate observations.

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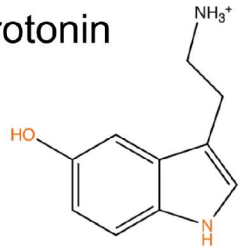
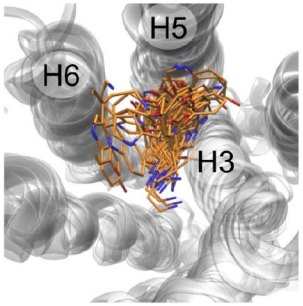
FIGURE 1

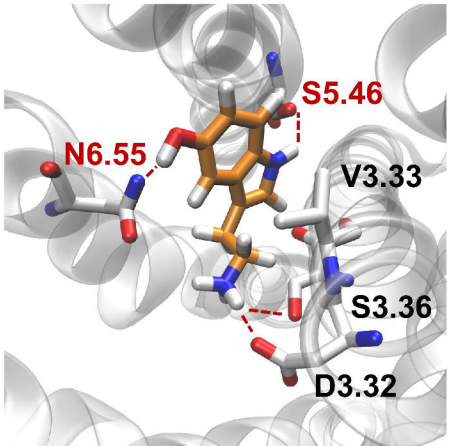
Serotonin



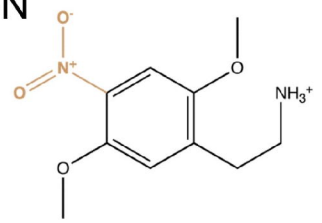
Conformational space analysis



Preferred ligand interactions



2C-N



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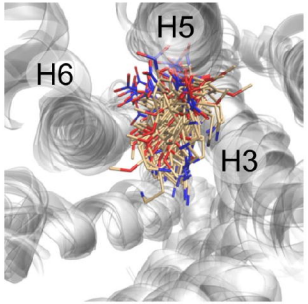


Preferred ligand interactions

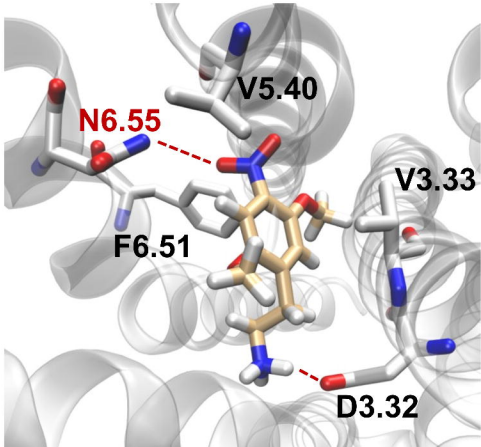


FIGURE 2

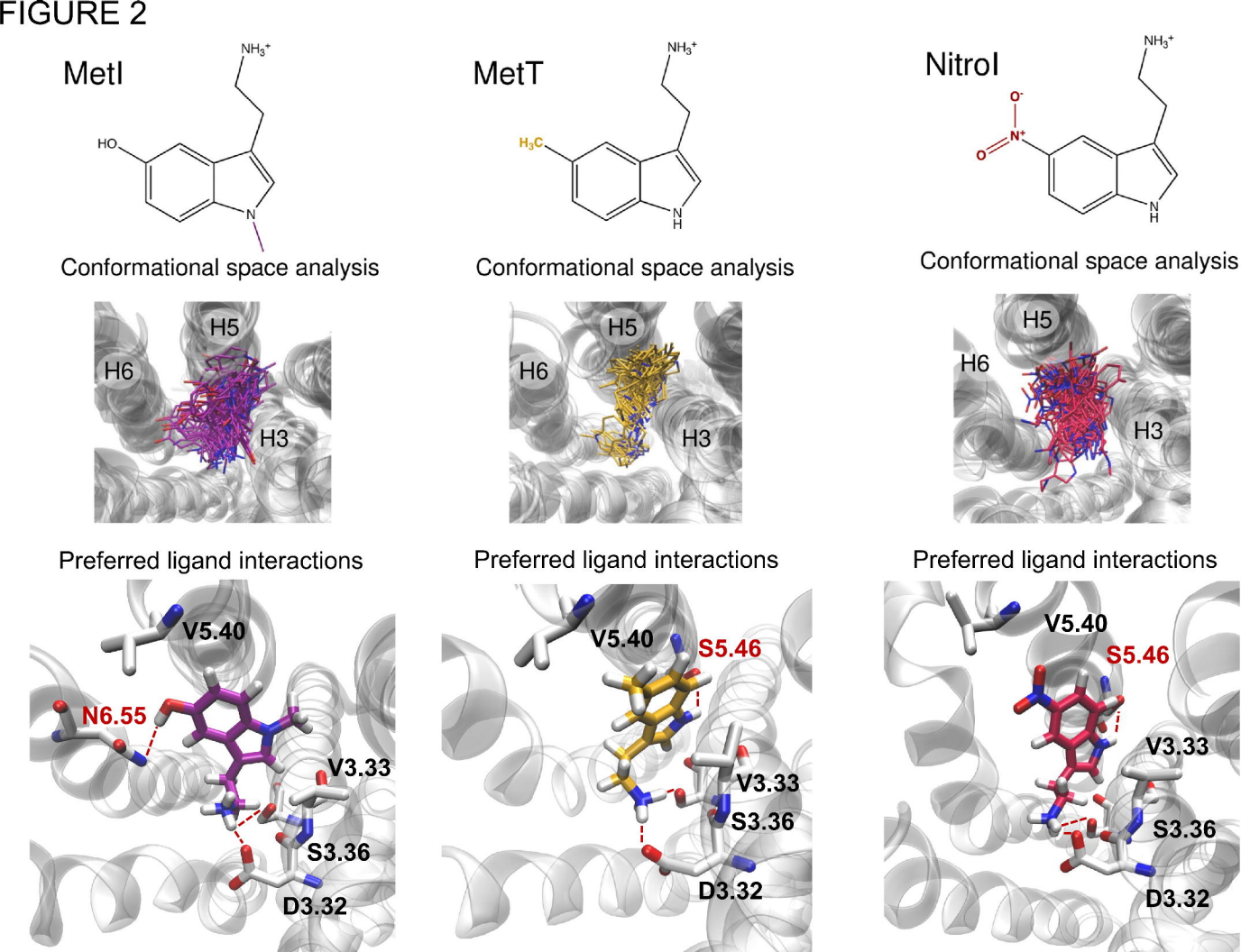
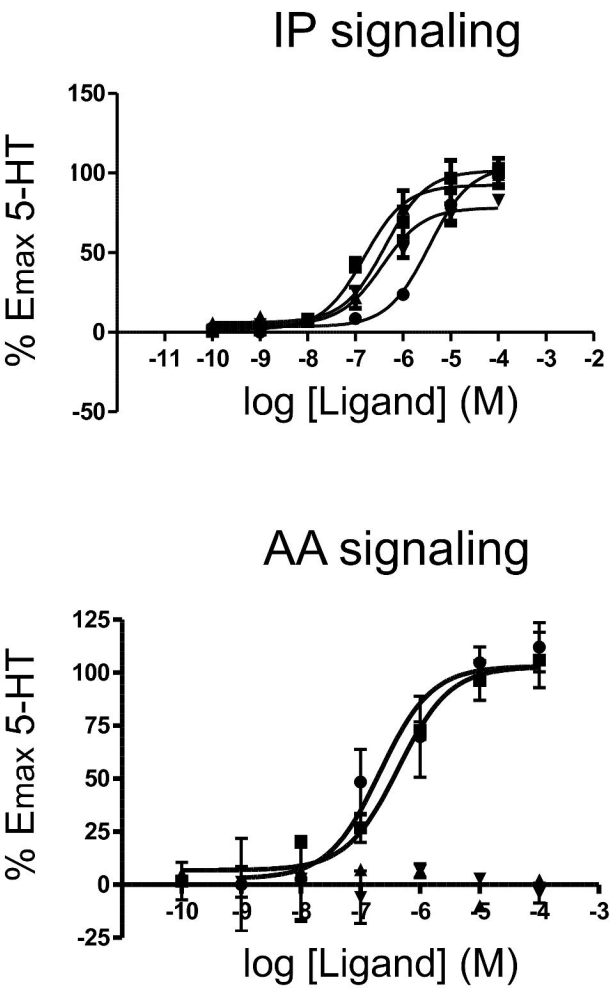

MetI
MetT
NitroI
Conformational space analysis
Conformational space analysis
Conformational space analysis
H5
H6
H3
H5
H6
H3
H5
H6
H3
Preferred ligand interactions
Preferred ligand interactions
Preferred ligand interactions
V5.40
N6.55
V3.33
S3.36
D3.32
V5.40
S5.46
V3.33
S3.36
D3.32
V5.40
S5.46
V3.33
S3.36
D3.32

FIGURE 3


IP signaling
% Emax 5-HT
150
100
50
0
-50
-11 -10 -9 -8 -7 -6 -5 -4 -3 -2
log [Ligand] (M)
AA signaling
% Emax 5-HT
125
100
75
50
25
0
-25
-10 -9 -8 -7 -6 -5 -4 -3
log [Ligand] (M)
5-HT
MetI
NitroI
MetT